

IN VITRO PROPAGATION OF *CEREUS PERUVIANUS* MILL. (CACTACEAE)

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(Received 5 March 1994; accepted 6 September 1994; editor R. H. Smith)

SUMMARY

Cereus peruvianus seedlings were used as a source of stem explants to determine the effective conditions for inducing and maintaining callus tissues in a state of rapid growth, as well as to obtain plants regenerated from callus cultures. Factorial combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin in MS medium were tested, and we concluded that the 18.1 μM 2,4-D and 18.6 or 27.9 μM kinetin combinations were suitable for callus induction. The cactus shoots were produced from the friable callus; root elongation occurred within 2 wk in medium without 2,4-D and with 18.6 μM kinetin. This method can be used to rapidly produce many *C. peruvianus* plants.

Key words: cactus micropropagation; friable callus; seedling stem explants; shoot formation.

INTRODUCTION

The classic methods of propagation of succulents plants are by seeds, cutting, and grafting (Krulik, 1980). Among members of the *Cereus* genus propagation from cuttings are useful. *Cereus peruvianus* is a species of medicinal and economic industrial importance (Scheinvar, 1985; Turbak, 1979; Alvarez et al., 1992) which exhibits very slow growth, and propagation by seeds is difficult due to poor germination.

Many species of cacti have been micropropagated by rooting of shoots proliferated through axillary branching in vitro (Mauseth, 1976; Johnson et al., 1976; Johnson and Emino, 1979; Vyskot and Jará 1984; Starling, 1985; Ault and Blackmon, 1987; Escobar et al., 1986; Clayton et al., 1990; Smith et al., 1991). However, *C. peruvianus* micropropagation through axillary branching was difficult. The in vitro culture of axillary buds of adult plants result in a high level of contamination, and a low number of plantlets were obtained from seeds germinated in vitro (Oliveira, 1993).

Thus, the present study reports the effective conditions for inducing and maintaining callus tissue of *C. peruvianus*, as well to regenerate whole plants.

MATERIALS AND METHODS

Shoot segments of adult plants were initially used as explants for callus induction, but the callus grew slow, and callus did not regenerate at any hormonal combination yet tested (Oliveira, 1993). Seeds of one *C. peruvianus* plant were sterilized in 70% ethanol for 5 min and in 5% sodium hypochlorite for 30 min, washed at least 3 times (10 min per wash) in sterile distilled water, and incubated aseptically in 0.8% agar-water medium under a 30 $\mu\text{Mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ cold-white fluorescent light (PPF) with photoperiod of 16 h at 28° C.

The germination occurred within 15 to 20 days (15% germination ratio), and after 8 wk the seeds developed into plants that were 2 to 3 cm tall and

lacked axillary buds. These plants were used as a source of explants (Fig. 1). Transverse segments approximately 2 to 3 mm wide and 1 to 3 mm in diameter were cut from the hypocotyl, and the explants were then placed in petri dishes containing 0.8% agar medium with 3% sucrose, prepared according to Murashige and Skoog (1962), containing B5 vitamins (Gamborg et al., 1968), plus 15% coconut water and a factorial combination of 2,4-dichlorophenoxyacetic acid (2,4-D) and *N*-(2-furanylmethyl)-1H-purin-6 amine (kinetin). Two experiments were replicated 3 times in one of each factorial combination 0, 5.0, 10.0, 15.0 μM 2,4-D and kinetin, and 0, 9.05, 18.1, 27.15 μM 2,4-D and 0, 9.3, 18.6, 27.9 μM kinetin. Petri dishes containing 12-stem explants were cultured in an incubator (B.O.D. mod. 347G) at 32° C under a 16-h photoperiod (15 $\mu\text{Mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PPF).

Shoot rooting did not occur in the absence of both 2,4-D and kinetin, and factorial combinations 0, 9.05, 18.1 μM 2,4-D and 0, 9.03, 18.6 μM kinetin were tested for root formation. The flats containing shoots in fresh medium were kept in the culture room with 30 $\mu\text{Mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PPF, 16-h photoperiod daily at 32° C.

RESULTS

After 4 weeks, all the explants cultured in 18.1 μM 2,4-D plus 18.6 or 27.9 μM kinetin developed green callus tissue, whereas the explants maintained on other combinations (0, 5.0, 10.0, 15.0 μM 2,4-D and kinetin, and 0, 9.5, 27.15 μM 2,4-D) gradually became necrotic and died within 8 weeks.

During the next 4 wk the callus was grown without subculture. Subculture to fresh medium containing 2,4-D 18.1 μM and kinetin 18.6 μM or 27.9 μM , with only 10% coconut water, was performed at 15-day intervals thereafter. After 12 wk, two types of callus were observed: one consisting of compact tissue and the other very friable tissue. The friable callus after 18 to 20 wk of culture produced small cactus shoots (Fig. 2), and the cultures of the compact callus continued growing but did not form shoots. These observations were evident in both combinations, but an independent test indicated association between culture medium and the callus type produced (Table 1). The 18.1 μM 2,4-D and 18.6 μM kinetin combination produced the most friable callus compared with 18.1 μM 2,4-D and

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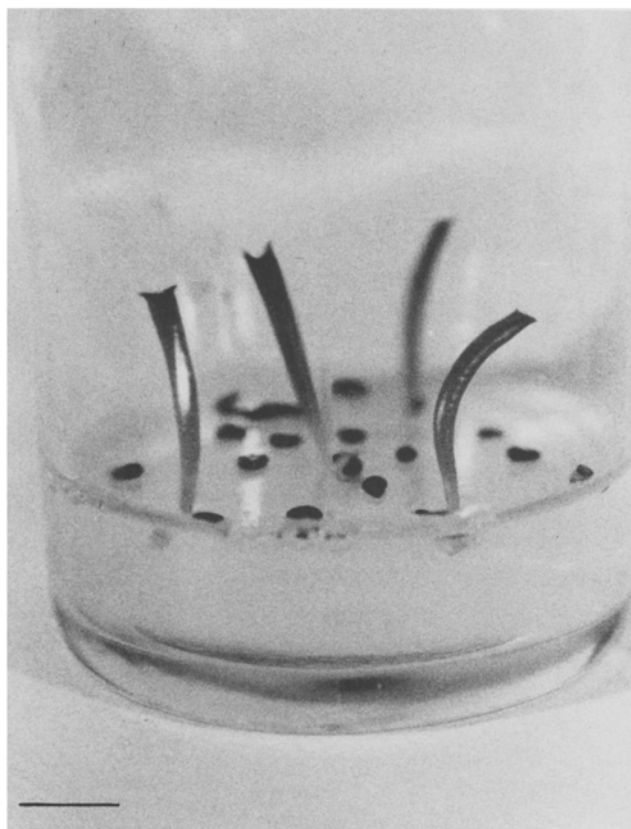


FIG. 1. *Cereus peruvianus* seedlings in 0.8% agar-water medium, with stems free of axillary buds which were cut in transverse segments and used as a source of explants for callus formation (bar = 10.0 mm).

27.9 μM kinetin. A single friable callus (10 to 20 mm) produced from 10 to 50 cactus shoots (Fig. 3). On average, 13.9 ± 6.5 shoots/callus were produced in 18.1 μM 2,4-D and 18.6 μM kine-

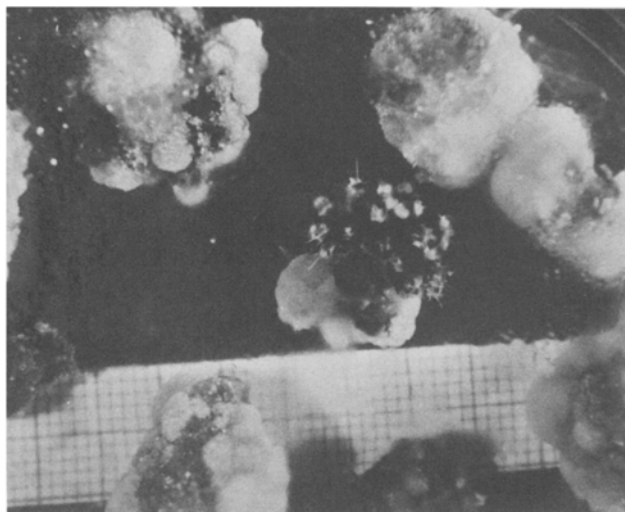


FIG. 2. Petri dishes containing *C. peruvianus* friable callus tissues and a cluster of new shoots (arrow) arising from the friable callus after 18 wk of subcultivation in medium containing 18.1 μM 2,4-D and 18.6 μM kinetin.

TABLE 1

ASSOCIATION BETWEEN CULTURE MEDIUM AND THE CALLUS TYPE PRODUCED (FRIABLE OR COMPACT) AND THE AVERAGE OF THE *C. PERUVIANUS* SHOOTS PRODUCED FROM CALLUS IN THE 2,4-D 18.1 μM AND KINETIN 18.6 OR 27.0 μM COMBINATIONS^a

2,4-K $\times$ Kinetin Combinations (μM)	Number of the Callus Produced		χ^2	Average of Shoots/Callus	t
	Friable	Compact			
18.1 $\times$ 18.6	157	59	33.01**	13.9 ± 6.5	2.35*
18.1 $\times$ 27.9	104	123		17.3 ± 7.9	

^a χ^2 : independence test, $P < 0.01$; t: $P < 0.05$, df 98.

tin, and 17.3 ± 7.9 shoots/callus in 18.1 μM 2,4-D and 27.9 μM kinetin. The 18.1 μM 2,4-D and 27.9 μM kinetin hormonal combination was most effective for shoot regeneration as compared to 18.1 μM 2,4-D and 18.6 μM kinetin (Table 1).

Well-differentiated cactus shoots, 1 to 4 cm long without roots, were then transferred to flats containing fresh medium (the same used for callus induction and subculture) and within 2 wk rooting percentage was 100% in medium with 18.6 μM kinetin and no auxin 2,4-D, whereas very few roots were observed in the shoots maintained on the other combinations. Thereafter, all shoots maintained on the other combinations were transferred to the medium with 18.6 μM kinetin and without 2,4-D, and within 4 wk the rooting percentage of these shoots was 96%. Root formation has occurred in medium lacking auxin, but root elongation was inhibited after 2 wk of root formation. To overcome this problem, the plants with root induction were placed in medium containing 9.05 μM 2,4-D and 18.6 μM kinetin, and maintained for 4 to 5 wk until roots elongated.

The cactus plants were transferred to pots containing a mix of red earth (Paleudult) and ground river sand (1:1). The pots were covered with plastic bags to prevent desiccation and allow acclimatiza-

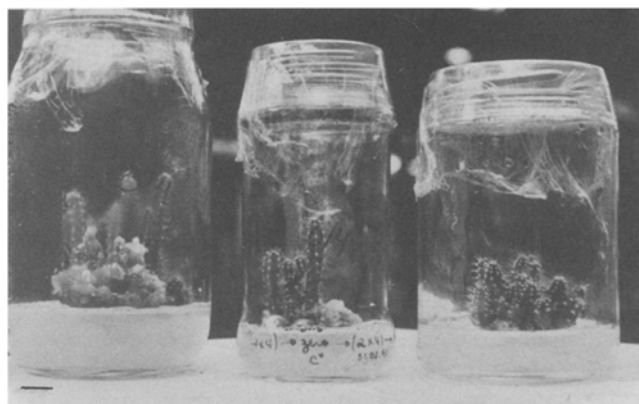


FIG. 3. Well-differentiated *C. peruvianus* cactus shoots regenerated from callus culture after 20 wk of subculture. At this stage, the multiple shoots were transferred to a medium containing 18.6 μM kinetin and without 2,4-D until the root development (4 wk) and then transferred to 9.05 μM 2,4-D and 18.6 μM kinetin for root elongation (bar = 5.0 mm).

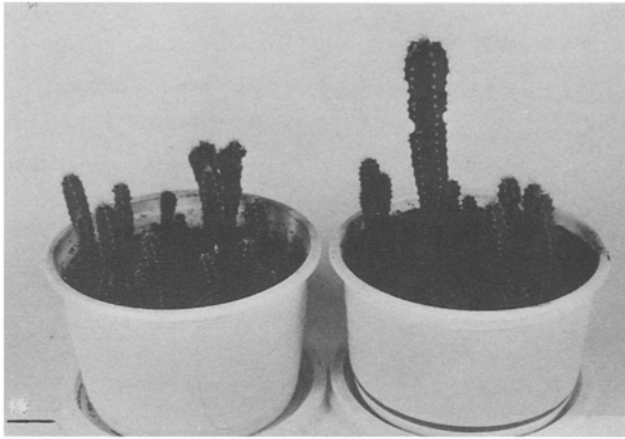


FIG. 4. Cactus plants (*C. peruvianus*) maintained at room temperature in a potting mix (1:1) of a red earth (Paleodult) and ground river sand (bar = 10.0 mm).

tion. Approximately 87% (3464/3983 shoots) of the plants survived and continued to grow (Fig. 4).

DISCUSSION

The auxin concentration used for callus induction in this study was high compared to that reported for callus induction in other cactus species (Smith et al., 1991), but we found that treatment with 5.0, 10.0 and 15.0 μM concentrations of 2,4-D were not effective for callus formation in *C. peruvianus*. In contrast, relatively high amounts of 2,4-D were required (10 to 20 mg/liter or 45.25 to 90.5 μM) for initiation of callus or for its continued proliferation in the *Neomammillaria profilera* species (Minocha and Mehra, 1974). We conclude that the MS medium, with B5 vitamins, plus 15% coconut water and 18.1 μM 2,4-D combined with 18.6 μM or 27.9 μM kinetin, can be used for callus induction and maintenance. The 18.1 μM 2,4-D and 18.6 μM kinetin combination produced the most friable calli, but on the other hand the 18.1 μM 2,4-D and 27.9 μM kinetin combination was most effective for shoot regeneration. Therefore both combinations can result in plant regeneration from callus cultures.

Coconut water prevented immediate browning of the explants because in preliminary experiments, over identical conditions without coconut water we observed browning of all the explants within 2 days after incubation. Coconut water or milk is usually added to the culture medium of some species where the initial explants produce excessive amounts of phenolic substances whose oxidation products darken both the tissue and the medium (Dixon, 1985). In cactus tissues culture, the optimum levels of coconut milk for callus formation were 10% (Steinhart, 1962) and 20 to 60% (Minocha and Mehra, 1974). For *C. peruvianus* species, 15% coconut water was initially necessary; however, coconut water seems unnecessary for maintenance during subsequent subcultures because the 10% concentration was sufficient in the five initial subcultures, and in subsequent subcultures callus tissues continued to grow in the absence of coconut water. In *N. profilera*, callus formation from different sources was also dependent on the interaction of 2,4-D, coconut milk, and kinetin, but coconut milk was required at all times for continued proliferation of callus on subculturing (Minocha and Mehra, 1974).

Rooting of shoots usually is achieved by subculturing to medium

lacking cytokinin with or without auxin (Hussey, 1986), but in contrast, root formation in *C. peruvianus* shoots occurred in medium with 18.6 μM kinetin and lacking 2,4-D. Other auxins considered effective to obtain active rhizogenesis with or without cytokinin were not tested because the rooting percentages were 100% in kinetin medium and no auxin. In contrast to the other cactus species (Mauseth and Halperin, 1975; Martínez-Vásquez and Rublo, 1989), lower auxin/cytokinin levels induced root elongation in the *C. peruvianus* shoots.

Thus, the comparison of results with tissue cultures of the different cactus species indicate similarities of some basic requirements such as 2,4-D, kinetin, and coconut milk or water, but no regularity in the respective concentrations of these nutritional requirements for growth in vitro.

The *C. peruvianus* plants regenerated from callus cultures may generate somaclonal variation (Larkin and Scowcroft, 1981; Karp and Bright, 1985). The implications of genetic instability of callus tissues culture have been reviewed by Meins (1986) and Hussey (1986). The study of isozyme patterns in callus cultures and in plants regenerated from calli of *C. peruvianus* showed that any isozyme induced in callus tissues could be used as markers for the culture conditions and for plants regenerated from callus tissues (Machado et al., 1993; Mangolin et al., 1994a,b). However, a low proportion (10.6%) of *C. peruvianus* plants showing all the isozymes induced in callus tissues from long-term culture indicates a transient expression of some of the induced isozymes (Prioli et al., 1994).

Therefore, callus induction and maintenance methods here related can be used to maintain germplasm in culture and to rapidly produce many *C. peruvianus* plants. Hypocotyl segments of six plantlets used as explants for callus induction at the two 2,4-D and kinetin combinations (18.1 μM 2,4-D $\times$ 18.6 μM kinetin and 18.1 μM 2,4-D $\times$ 27.9 μM kinetin) produced 3464 plants. Therefore, this method seems effective for in vitro propagation of *C. peruvianus* species.

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